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INTERNATIONAL APPLICATION PUBLISHED

WO 9602567A1

(51) International Patent Classification 6 :
C07K 14/47, A61K 38/17 A1

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/EP95/02723

(22) International Filing Date: 12 July 1995 (12.07.95)

(30) Priority Data:
MI94A001469 14 July 1994 (14.07.94) IT

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(74) Agent: BIANCHETTI, Giuseppe; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROTEINS FROM MAMMALIAN LIVER AND THEIR USE IN ONCOLOGY

(57) Abstract

The present invention refers to proteins extractable by perchloric acid from mammalian liver, particularly from goat liver, and to their use in oncology.

FOR THE PURPOSES OF INFORMATION ONLY

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PROTEINS FROM MAMMALIAN LIVER AND THEIR USE IN ONCOLOGY

The present invention refers to proteins from animal tissues, particularly from mammalian liver, and to the use thereof in oncology.

WO 92/10197 discloses extracts of mammalian organs, particularly of goat liver, consisting of at least three different proteins and characterized by unusual pharmacological and immunological properties. No information was reported on the actual role and on the sequences of the individual protein components.

A 23-KDa dimeric protein extracted with 5% perchloric acid from rat liver and kidney has been disclosed in Eur. J. Biochem. 272, 665, 1993. The corresponding cDNA sequence have been deposited at the EMBL data bank under accession number X70825.

This protein, reported to be co-extracted with High-mobility group (HMG) proteins, is suggested to play a role in the folding of proteins, so that it could be considered as one member of the class of the so-called "chaperons" or chaperonins.

WO 93/18146 discloses a protein extracted from rabbit-liver having a molecular weight of 59Kd capable of complexing with chaperons and a heat shock protein of 90 Kd.

A new protein purified from the extract disclosed in WO 92/10197, has been found now having the partial aminoacid sequence depicted in sequence Id n. 1.

Said protein is useful in oncology in view of the following properties:

- the serum of animals immunized with the protein displays cytotoxic activity against human tumor cell cultures;
- the protein has marked antineoplastic activity at the dose of 0.015 µg/kg in Balb/c mice having a murine colon adenocarcinoma (c26) and in rats with intrapleuric Yoshida ascitic tumor;
- when administered to animals, man included, it raises antibodies able to recognize human carcinoma cells.

Said properties explain the activity observed in clinical tests carried out administering the extract of WO 92/10197 to patients affected by advanced cancer of the lung, breast, stomach, colon and liver.

The protein of the invention has a high degree of homology with that extracted from rat liver disclosed in Eur. J. Biochem. 272, 665, 1993.

Proteins having a high degree of homology with that of Sequence Id n. 1 have also been found in liver of different animal species, particularly bovine and equine liver. The invention refers also to said homologous sequences, except the known sequence from rat liver.

A new protein family has been therefore found: the members of this previously unknown family are characterized by an high degree of conservation and homology between the mammalian species and a molecular weight ranging from about 10 KDa to about 14 KDa.

The term "high degree of homology" means an homology of the aminoacid sequences of about 80% or higher, preferably of 90% or higher.

The invention further refers to the use in oncology, as a therapeutic and/or diagnostic tool, of the above mentioned perchloric acid extractable proteins from mammalian liver.

The invention provides therefore pharmaceutical compositions containing the protein having the partial amino acid sequence n. 1 or proteins having at least 80% homology, preferably at least 90% homology, with Sequence Id n. 1.

The pharmaceutical compositions of the invention will be administered by parenteral route, preferably subcutaneously or intra-muscularly and will typically contain from 0.1 to 50 mg of total protein per unit dose. The protein active principle, purified by conventional methods, may be lyophilized on a suitable non-toxic carrier and distributed in vials or bottles.

Suitable solvents include sterile water or saline solutions.

According to a further embodiment of the invention, the proteins of the invention or fragments thereof, produced for instance by chemical synthesis, may be used to produce polyclonal or monoclonal antibodies. Particularly interesting antibodies recognize tumoral antigens and are therefore useful for diagnostic, therapeutic or research purposes. Two of said antibodies have been deposited on 27-7-1993 at the European collection of Animal Cell Cultures (EGACC), Porton Down, Salisbury, UK under accession numbers 930806103 and 930806104.

These antibodies were used in immunocytochemical tests on several bioptic samples of human cancers,

enabling their recognition.

The proteins of the invention, when administered to patients affected by neoplastic disease, in addition to advantageous effects such as inhibition or regression of the tumoral mass, reduction of pain and improvement of cenestesis, raise antibodies having marked cytotoxic action on cultured tumor cells. The whole serum, not free from the complement cascade, is required for said cytotoxic effect.

When used for therapeutic purposes or as a vaccine to induce immunity against neoplastic transformation, the proteins of the invention may be administered at a dosage ranging from 0.1 to 30 mg/day/patient, by the subcutaneous, intramuscular or intravenous route. The treatment will be repeated even for long periods, until the concentration of the raised antibodies reaches a convenient level.

The concentration of the raised antibodies may be determined by conventional methods, using for instance immunoenzymatic techniques. To this purpose, the invention provides diagnostic kits containing suitably labelled reagents, e.g. the protein of the invention or fragments thereof as an antigen, optionally immobilized on a suitable support, anti-Ig antibodies and suitable reagents able to detect, e.g. by means of a colorimetric reaction, an antigen-antibody complex.

The proteins of the invention may advantageously be administered together with suitable carriers, acting as adjuvants. Suitable adjuvants may be selected, for instance, from non-toxic proteins, preferably xenogenic proteins, e.g. proteins from the same species from

which the immunogenic protein is extracted.

The proteins of the invention are prepared by subjecting the crude extract, obtained by extracting the organs with perchloric acid and subsequently with hypertonic saline solutions (KCl 3M for instance) and subsequent dialysis, to purification steps in HPLC and hydrophobic exchange chromatography (FPLC) as hereinafter specified in the Examples.

The protein obtained from goat liver is blocked at the N-terminal and it has been therefore partially sequenced after cleavage with CNBr, yielding two main fragments having molecular weight (determined by the MALDI-TOF method) respectively of 10263 and 4063 D, respectively, whereas the molecular weight before cleavage is 14.290 Daltons, in agreement with the value determined by SDS-PAGE electrophoresis.

The following examples further illustrate the invention.

Example 1

A liver goat extract, prepared as in WO 92/10197, and hereinafter referred to as UK 101, is concentrated on Amicon PM 10 membrane and subsequently dialyzed against NaH_2PO_4/Na_2HPO_4, 0.01 M, pH 6.5. The product is purified by HPLC on TSK DEAE 5 PW equilibrated in said buffer; the starting buffer is collected and the protein absorbed on the resin are eluted with 1M NaCl. The peak eluted in the starting buffer is subsequently purified by HPLC on TSK SW 3000 column.

Two main peaks are obtained by this chromatography: the first is discarded since it mainly consists of glycogen; the second, particularly rich in

low molecular weight proteins, is then purified by FPLC on Protein-Pac HIC Phenyl 5 PW column.

The purification on this hydrophobic exchange column, is carried out in the following conditions: a starting buffer, Tris HCl 20 mM pH 7 containing $(NH_4)_2SO_4$ 1M, is first eluted, followed by a linear gradient elution ending with Tris- HCl 20 mM without ammonium sulfate. The starting buffer is discarded whereas the zone, eluted in the gradient at a $(NH_4)_2SO_4$ molarity ranging from 0.6 to 0.8 M is collected and dialyzed against H_2O.

A sample hereinafter referred to as UK 114 showing a protein band in SDS-PAGE of about 14 Kda with a purity degree of about 90% is obtained.

Example 2

In immunocytochemical tests, polyclonal antibodies raised in rabbits immunized with liver goat extract (WO 92/10197) administered subcutaneously in PBS with Freund's complete adjuvant every week for 2 months were used.

Monoclonal antibodies were obtained from Balb/c mice one month after weekly subcutaneous injections of 100 µg of UK 101 with incomplete Freund's adjuvant. The fusion with myeloma cells of lymphocytes obtained from animals immunized against UK 101 was carried out by conventional methods. Two of the obtained hybridomas were deposited on 27-7-1993 at the European Collection of Animal Cell Cultures (ECACC) Porton Down, Salisbury, UK, under accession numbers 930806103 and 930806104.

The antibodies secreted by said hybridome

recognize the proteins of the invention.

The mono- and polyclonal antibodies have been assayed in immunocytochemistry tests on 30 bioptic samples of malignant tumors isolated from different organs such as breast, lung, bladder, stomach, colon-rectum, uterus, soft tissues, prostate. The tissues were fixed in 10%, buffered formaline and preparations in paraffine were stained by means of Mistostain Kit SP, Zymed Lab. Inc..

The sections were incubated with the antibodies (0.5 µg/ml of Ig with 1% BSA/PBS) overnight at 4°C. After washing, the slides were incubated with anti-rabbit pig biotinylated Ig for 60 minutes and then for other 60 minutes with a 1:100 dilution of peroxidated streptavidine-biotine complex. The peroxidase binding was detected using the 3,3-diaminobenzidine/H_2O_2 reaction. Only the tissue showing specific reaction against the antibodies in the cytoplasma were considered positive. The immunoreactivity was considered as negative, slightly positive, positive (++) and highly positive (+++) for the normal tissues. The results are reported in the following Table. The immunocytochemical reactivity with different polyclonal antibodies anti goat, calf and horse liver extract is detectable in most malignant tumors (82.7% for antibodies against horse liver extract and 100% for calf liver extract). The monoclonal antibody secreted by the hybridoma n. 930806103 gave positive results for 93.7% of the assayed tumors.

TABLE

Immunocytochemical reactivity of malignant tumors (+/-)

	Anti UK101			
SITE	Goat	Calf	Horse	Mab n. 930806103
Breast	4/0	1/0	1/0	1/0
Stomach	4/3	3/0	3/0	3/0
Colon/rectum	7/0	5/0	5/0	5/0
Lung	1/1	n.a.	n.a.	1/0
Bladder	2/0	1/0	1/0	1/0
Prostate	3/0	1/0	1/0	1/1
Uterus	1/0	1/0	1/0	1/0
Adrenal gland	1/0	1/0	1/0	1/0
NOS	2/1	1/0	0/1	1/0
Total	25/5	14/0	13/1	15/1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZETESIS
(B) STREET: Galleria del Corso 2
(C) CITY: MILAN
(E) COUNTRY: ITALY
(F) POSTAL CODE (ZIP): 20122

(ii) TITLE OF INVENTION: PROTEINS FROM MAMMALIAN LIVER AND THEIR USE IN ONCOLOGY

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Capra hircus
(F) TISSUE TYPE: Liver

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Asp Pro Ala Ser Gly Gln Leu Val Pro Gly Gly Val Val
1 5 10
Glu Glu Ala Lys Gln Ala Leu Thr Asn Ile Gly Glu Ile Leu
15 20 25
Lys Ala Ala Gly Xaa Asp Phe Thr Asn Val Val Lys Ala Thr
30 35 40
Val Leu Leu Ala Asp Ile Asn Asp Phe Xaa Ala
45 50

INTERNATIONAL FORM

TO

Zetesis spa
Galleria del Corso
2 Milano
Italy

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

P3D1D11

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

930806103

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 06.08.93 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Dr A Doyle
ECACC
CAMR

Address:

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 8 March 1994

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

Zetesis spa
Galleria del Corso
2 Milano
Italy

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Zetesis spa Address: Galleria del Corso 2 Milano Italy	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: 930806103 Date of the deposit or of the transfer: 6 August 1993
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 6 August 1993 [2]. On that date, the said microorganism was [X] [3] viable [ ] [3] no longer viable	

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr A Doyle ECACC Address: CAMR	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 8 March 1994

[4] Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. Proteins extractable from mammalian liver but not from rat liver having the partial aminoacid sequence of Sequence Id n. 1 or sequences having an homology degree of at least 80% with said Sequence Id n. 1.

2. Proteins according to claim 1 having an homology degree of at least 90% with said Sequence Id n. 1.

3. Proteins according to claim 1 or 2 extractable from goat, horse or calf liver.

4. Proteins according to claim 3 extractable from goat liver.

5. Proteins according to any one of the previous claims having molecular weight from about 10 to about 14 Kda.

6. Proteins according to claim 5 having molecular weight of about 14 Kda.

7. Proteins according to any one of the previous claims, recognized by the antibodies secreted from the hybridomas deposited at ECACC under numbers 930806103 and 930806104.

8. Proteins extractable from mammalian liver having the partial aminoacid sequence of Sequence Id n. 1 or sequences having an homology degree of at least 80% with said Sequence Id n. 1, for use in anti-tumor therapy.

9. Pharmaceutical compositions containing as the active principle the proteins of claim 1 or 8 in admixture with a suitable carrier.

10. Use of the proteins extractable from mammalian liver having the partial aminoacid sequence of Sequence

Id n. 1 or sequences having an homology degree of at least 80% with said Sequence Id n. 1 in diagnostics.

INTERNATIONAL SEARCH REPORT

Internat. Application No
PCT/EP 95/02723

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 10197 (BARTORELLI ALBERTO ;TURIANO ANGELA (IT)) 25 June 1992 cited in the application see claims; examples ---	1-10
X	EUR. J. BIOCHEM. (1993), 212(3), 665-73 CODEN: EJBCAI;ISSN: 0014-2956, 1993 LEVY-FAVATIER, FLORENCE ET AL 'Characterization, purification and cDNA cloning of a rat perchloric-acid-soluble 23-kDa protein present only in liver and kidney' cited in the application see page 665, left column, paragraph 1 - right column, paragraph 1; figure 3 see page 672, left column, paragraph 2 - right column, paragraph 1 --- -/--	1-5,7,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 1995	05.12.1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internat ·: Application No
PCT/EP 95/02723

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE EMBL Emrod:Rspspl; Access-no: D49363 OKA, T. 'Sequence of PSP1'; 22 February 1995 see abstract ---	1-6,8
P,X	DATABASE EMBL Emest:Hs68065, Access-no: T98680 HILLIER, L. ET AL 'The WashU-Merck EST Project'; 17 April 1995 see abstract -----	1-6,8

1

INTERNATIONAL SEARCH REPORT

In...national application No.
PCT/EP 95/02723

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat l Application No
PCT/EP 95/02723

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9210197	25-06-92	AT-T-	122890	15-06-95
		AU-B-	661287	20-07-95
		AU-B-	9035791	08-07-92
		CZ-A-	9301116	13-04-94
		DE-D-	69110060	29-06-95
		DE-T-	69110060	28-09-95
		EP-A-	0574394	22-12-93
		HU-A-	64569	28-01-94
		JP-T-	6504039	12-05-94